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Identification of Salt Stress Responsive Protein in *Lactobacillus Paracasei* LN-1 Using SDS-PAGE

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Abstract

Lactic acid bacteria (LAB) are widely used in fermentation industry, not only because LAB can form the food texture and key flavor, but also due to their contribution to the health of human. During the industrial processes, LAB always confront with different environmental stress, including salt stress. In the present study, salt stress response of a potential probiotic bacterium *Lactobacillus paracasei* LN-1 which isolated in Chinese traditional fermented food *suan-cai* was examined using SDS-PAGE at the protein level. The results showed that a small heat shock protein was found that induced by 6.5% and 8.0% NaCl, probably indicating the link between the protein and osmotic tolerance of the strain. The study also detected the expression level of *hsp60* and *hsp 70* genes of the strain using RT-PCR under salt stress.

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1. Introduction

Lactic acid bacteria (LAB) have been ubiquitously used as starters in fermentation industry to form the food texture and key flavor. Lactic acid produced by LAB is also take part in inhabitation the growth of spoilage strains. Moreover, the importance property of LAB is regarded as safe starter, due to their contribution to the healthy of human (Stiles et al., 1996). Therefore, the interesting in LAB is rising.

During the industrial production, LAB always confront with different environmental stress, such as heat, cold, acid, and salt stress, etc., which influence the metabolism of LAB. In most of previous studies of LAB, heat shock proteins (HSPs) were always identified in response to the environment stress. The further studies indicated that HSPs have the ability to protect other proteins from damage by environmental stress and to assist in normalization of cellular functions during recovery from stress (Nover et al., 1997). HSPs are belong to molecular chaperones, including Hsp 60 (GroEL), Hsp70 (DnaK), Hsp 100, and so on. They are a class of functionally related proteins involved in protein folding and protein turnover. Recently, protein and genes of GroEL and DnaK have been identified in LAB whose expressions were induced in stressful conditions. In *Lactobacillus helveticus* PR4, GroEL and DnaK were identified to heat stress (Di Cagno et al., 2006). GroEL and DnaK were also found unregulated in *Lactococcus lactis* MG1363 cultured in chemically defined medium at pH 5.0 (Budin-Verneuil et al., 2005). While, Kilstrup et al (1997) also identified the induction of GroEL and DnaK in *L. lactis* MG1363 under salt stress.

In this study, we aimed to examine the salt stress response of a potential probiotic bacterium *Lactobacillu paracasei* LN-1 which isolated from *suan-cai* at the protein level using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). It would be helpful to achieve a better understanding of the impact of salt stress to LAB during food processing.

2. Materials and methods

2.1. Bacteria growth condition

In The bacterial strain used in the study was *L. paracasei* LN-1 which isolated from traditionally home-made *suan-cai* in Liaoning province of China (Wu et al., 2014). For salt stress response analysis, 1.0% (v/v) overnight suspensions of *L. pracasei* LN-1 were cultured into 100 mL fresh De Man, Rogosa, and Sharpe containing MES (2-(N-morpholino) ethanesulfonic acid) and MOPS (3-(N-morpholino) propanesulfonic acid) (MRS-M) broth with 0%, 3.0%, 6.5%, 8.0% NaCl (w/v), and incubated until the density at 620 nm reached 0.8 (mid-log phase, approximately 3.2×10^8 CFU/mL). Then appropriate cultures were collected by centrifugation at 4, 000 ×g at 4 °C for 5 min for protein extraction.

2.2. Protein extraction

The proteins were extracted using sonication method with some improvements (Gonzalez-Marquez et al., 1997). Culture samples were suspended in lysis buffer containing 8 M urea, 60 mM DTT, 4% CHAPS, and 1 mM PMSF. Then the solution was sonicated for 5 min as ultrasonic 30 s, pulse duration 30 s, power output 25 W. Ice-water was used to keep low temperature. The cell proteins were collected at 10, 000×g for 30 min by centrifugation. Soluble proteins in the supernatant were placed at –80℃ for further study. Bradford assay was used to detect protein concentration (Bradford 1976). Mini Protean 3 Cell electrophoresis apparatus was used in the study as described by Laemmli (1970).

2.3. SDS-PAGE

The SDS-PAGE consisted of 5% stacking gel and 12.5% separation gel. Sample volume was controlled to 20 μg proteins in each sample with 5 μL of 6×bromophenol blue. Electrophoresis was conducted at a constant current of 80V through the stacking gel and 120V through the resolving gel. R250 was used to stain the gel.

2.4. Analysis of gel and protein identification

Gel was scanned and analyzed through PDQuest 6.0 (Bio-Rad, USA). The differentially expressed protein was excised and following peptide trypsin performance according to Wu et al. (2009). The gel pieces were placed into 4700 Proteomics Analyzer (ABI, USA) for protein identification analysis. GPS ExplorerTM v3.5 software (ABI, USA) was used for the assay of mass spectrum data against the NCBInr database for firmicutes proteins.

2.5. Real-time PCR

EZNATM Bacterial RNA Kit (Omega Bio-tek, USA) was used in the study to extract total RNAs from cells, and RNase-free DNase-I (Omega Bio-tek, Inc) was added to final RNAs to digest genomic DNA. Further, cDNA was reverse transcribed by 100ng total RNAs using PrimeScript^{TM} RT reagent Kit (TaKaRa, Japan). The 10 μl reaction system included 0.5μl RT Enzyme Mix I, 1× buffer, and 50 pmol Random primer. Then, RT-PCR was performed. The 1 μl cDNA template was used in the 50μl extraction mixture, and 0.6×SYBR Green-I dye (Generay Biotech, China), 1× buffer, 0.3 u rTaq polymerase, 0.5 μM each primer, 0.2 mM dNTP mix were added. The procedure contained 40 cycles of amplification, including 94 ºC for 30 s, 63 ºC for 30 s, and 72 ºC for 30 s.

In PCR amplification, GAPDH was used as an internal control. The *hsp60* and *hsp70* primer pairs were designed based on genomic data of *L. paracasei* on NCBI are as follows, *hsp60* (upper: upper: 5'-CCTGACATTGAGAACCAAAATA-3', lower: 5'-TTGGAAGCCCATACCT ACGACT-3'), and *hsp70* (5'-TGACGATATGCACAACTATAGC-3', lower: 5'-GCATCCGATCAACAAGCGAGAG -3'). Melting curves for each PCR were carefully monitored to avoid nonspecific amplifications. Gene expression quantization was transformed by using the comparative Ct method.

3. Results and discussion

3.1. SDS-PAGE analysis

The Cellular proteins of *L. paracasei* LN-1 were extracted from mid-exponential-phase cells in MRS-M with 0%, 3.0%, 6.5%, 8.0% NaCl and resolved by SDS-PAGE (Fig. 1). The growth rate of *L. paracasei* LN-1 in MRS-M containing 3.0%, 6.5%, 8.0% NaCl was restrained at different degree, but the strain is able to grow in MRS-M with 3.0%, 6.5%, 8.0.% NaCl.

Comparing the protein profile, high visual and numerical similarity could be observed. The result showed that SDS-PAGE is suitable for resolve the proteins of LAB. Besides, SDS-PAGE also has been applied in identification of LAB involved in complicated resource (Kersters et al., 1980).

3.2. Different protein identification

Further comparison showed that there was a 17kDa protein spot found in *L. paracasei* LN-1 grown in MRS-M with 6.5% and 8.0% NaCl, whereas not appeared on gel of 0% and 3.0% NaCl. These small proteins are difficult to detect using standard biochemical techniques (Hemm et al., 2010). Based on the MS identification, we found small heat shock protein (sHSP) involved was induced by salt stress in *L. paracasei* LN-1. Moreover, the protein could be induced by higher concentration of NaCl over than 3.0%.

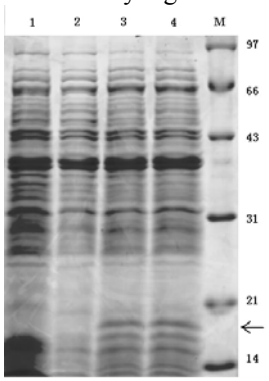

Fig. 1. SDS-PAGE of mid-log phase proteins of *L. paracasei* LN-1 cultured in MRS-M with 0% (1), 3.0% (2), 6.5% (3) and 8.0% (4) NaCl.

sHSPs are one of important low molecular members of HSPs. sHSPs are usually between 12-30 kDa. sHSPs are wildly distributed in bacteria and animals, except for some bacteria species, e.g. *Mycoplasma genitalium* (Narberhaus 2002). sHSPs are also crucial for the response to environmental stress in bacteria because of the capacity to act as ATP-independent chaperone. Chaperone can inhibit the polymerization of denatured proteins by stress, and promote the refolding of the denatured protein under optimal conditions to maintain the normal structure and function of protein (Ehrnsperger et al., 1997). Thus, many studies are now focusing on the expression and modification of sHSPs involved in LAB and other bacteria. O'Sullivan et al. previously reported that *Streptococcus thermophilus* cells had two genes of *shsp* in a plasmid which could resist to elevated temperatures (O'Sullivan et al., 1999). Moreover, in *S. thermophilus*, the pAGS4E encoding *shsp* has been proved to be able to protect cells against high acid, such as pH 3.5 (EI Demerdash et al., 2003). Recently, genome analysis has been widely used in the research of LAB. Based on the study of the genome sequencing analysis of *Bifidobacterium breve* UCC2003, a single sHSP-encoding gene hsp20 was found. Heat stress and osmotic shock could up-regulate the transcription of *hsp20* (Ventura et al., 2007).

Hence, the results of suggested that sHSP protein observed in *L. paracasei* LN-1might be linked to the osmotic functionality that the strain acquired. Further study is performing in our lab. It is noteworthy that sHSP protein is known to be involved in bacterial resistance to salt stress.

3.3. Real-time PCR of gene expression

In this study, we did further investigations to address the expression level of *hsp60* and *hsp70* in *L. paracasei* during salt stress using real-time PCR (Fig. 2).

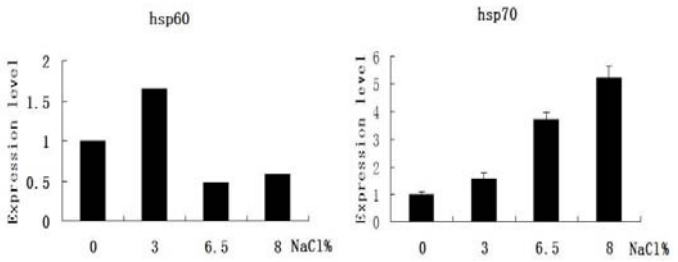

Fig. 2. Real-time PCR analyses of genes of *hsp60* and *hsp70* in *L. paracasei* LN-1 during growth in MRS-M with 0%, 3.0, 6.5 and 8.0% NaCl.

The transcriptional profiles of *hsp60* and *hsp 70* have different expression patterns. The expression of *hsp70* peaked at point 8.0% NaCl which were matched to the protein expression level, while the sharp increase expression of *hsp60* was found at 3.0%NaCl, then decreased at 6.5% and 8.0% NaCl. It might be associated with early translation and trafficking of stored mRNAs during the exponential phase. Further study is needed.

Acknowledgements

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